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(71) Applicant *(for all designated States except US)*: **INSTITUTE OF GENETICS AND DEVELOPMENTAL BIOLOGY, CHINESE ACADEMY OF SCIENCES** [CN/CN]; Datun Road, Chaoyang District, Beijing 100101 (CN).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CHU, Chengcai** [CN/CN]; c/o Institute of Genetics and, Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101 (CN). **SI, Lizhen** [CN/CN]; c/o Institute of Genetics and, Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101 (CN). **ZHANG, Yafang** [CN/CN]; c/o Institute of Genetics and, Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101 (CN). **TONG, Hongning** [CN/CN]; c/o Institute of Genetics and, Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101 (CN). **WANG, Yiqin** [CN/CN]; c/o Institute of Genetics and, Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101 (CN). **PANG, Xuebiao** [CN/CN]; c/o Institute of Genetics and, Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101 (CN). **HAJIREZAI, Mohanmad** [DE/CN]; c/o Institute of Genetics and, Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101 (CN). **SONNEWALD, Uwe** [DE/CN]; c/o Institute of Genetics and, Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101 (CN).

(74) Agent: **GUOQUN, Hu**; c/o CAWLEY, JR., Thomas A., Pillsbury Winthrop Shaw Pittman LLP, P.O. Box 10500, McLean, VA 22102 (US).

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(54) **Title:** EXPRESSION CASSETTES AND METHODS FOR INCREASING PLANT YIELD

(57) **Abstract:** An expression cassette and a method for improving plant yield are provided. The cassette comprises a mesophyll-specific promoter, a pyrophosphatase gene and a terminator, which are operably-linked. The method for improving plant yield comprises transforming the mesophyll-specific expression cassette of pyrophosphatase into plants, and screening for plants capable of expressing pyrophosphatase, then obtaining the plants with improved yields. The transgenic plant cell and whole plant produced by the method are also provided.

WO 2009/077973 A1

EXPRESSION CASSETTES AND METHODS FOR INCREASING PLANT YIELDS

[0001] The present invention claims the priority of CN 200710179540.0, filed December 14, 2007, the entire contents of which are specifically incorporated by reference for all purposes.

FIELD OF THE INVENTION

[0002] The present disclosure relates generally to methods of improving plant yields, and particularly, but not by way of limitation, to expression cassettes containing pyrophosphatase genes and mesophyll-specific expression promoters. The present invention relates to new plasmids and methods for the preparation of transgenic plants, as well as the plants, that are modified through the transfer and the expression of genes which influence the sugar metabolism or the sugar partitioning within a plant, and which are localized on these plasmids.

BACKGROUND

[0003] The information provided below is not admitted to be prior art to the present invention, but is provided solely to assist the understanding of the reader.

[0004] Plant growth, development, and yield depend on the energy that the plant gains by photosynthetic fixation of CO_2 as carbohydrates. The primary photosynthetic loci leaf and, to a lesser extent, stem tissues. Other organs, such as roots, fruits, seeds and tubers, do not make a material contribution to the formation of photoassimilates, but on the contrary are dependent for their growth on a supply of photoassimilates from photosynthetically-active organs. There is a flow of photosynthetically-gained energy from photosynthetically-active tissues to photosynthetically-inactive parts of a plant. Photosynthetically-active tissues are generally known as sources and are net exporters of fixed carbon dioxide. Photosynthetically-inactive parts of a plant are designated as sinks and are net importers of photosynthetically fixed carbon dioxide.

[0005] A fraction of the primary photoassimilates are converted into starch in chloroplast, and a second fraction is transported into the cytoplasm for sucrose synthesis and other triose-based metabolic reactions. In most plants, photoassimilates are distributed within a plant in the form of sugars and preferentially in the form of sucrose. The distribution of sucrose between the

source and sink tissues occurs by transport of sucrose via the phloem. One of the important determinants for the strength of a sink is the unloading of the phloem in the sink.

[0006] The efficiency of photosynthesis and partitioning of the two major primary photoassimilates, starch and sucrose, has a significant effect on grain yield. Currently, increasing grain yield is mainly affected by three factors: 1) increasing sucrose synthesis in source leaves; 2) increasing transportation ability of sucrose, i.e. increasing expression amount or enzymatic activity of H^+/sucrose transportation carrier; 3) improving the utilization of sink.

[0007] The rate of plant photosynthesis depends on light intensity, CO_2 concentration, etc., and may also be influenced by self-metabolic factors such as the activity of various CO_2-fixing Calvin cycle enzymes, *e.g.*, ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCo) and chloroplast fructose-1,6-diphosphate, partitioning of primary photoassimilates, transportation of carbohydrate assimilates, and the utilization of sink organs.

[0008] Material interchange between chloroplast and cytoplasm is important for modulating photosynthesis rate to meet the need of plant tissues for photosynthesis products, which is accomplished by chloroplast membrane system. A specific triose phosphate translocator (TPT) on inner chloroplast membranes is responsible for triose phosphate translocation. Under normal physiological conditions, TPT transport involves a strict 1:1 counter exchange, i.e. a molecule imported into the chloroplast is exchanged for another molecule exported from the chloroplast. Once exported from chloroplasts, triose phosphate is utilized in the cytoplasmic synthesis of sucrose, amino acids, and other molecules. The rate-limiting enzymes for cytoplasmic sucrose synthesis are cytoplasmic fructose-1, 6-bisphosphatase (cyFBPase) and sucrose phosphate synthase (SPS). The former, in combination with phosphofructokinase (PFK) and fructose-6-phosphate-1-phosphotransferase (PFPase), regulates the conversion of fructose-1,6-bisphosphate (FBP) to fructose 6-phosphate (F6P); the latter catalyzes the final step of sucrose synthesis. Altering the activities of these enzymes, or the balance of their reactions, allows the equilibrium to shift towards increasing sucrose synthesis. In the presence of inorganic phosphate (Pi), PFPase catalyzes the dephosphorization of FBP forming F6P and inorganic pyrophosphate (PPi). PPi is also released by the conversion of glucose-1-phosphate (G1P) to UDP-glucose in sucrose synthetic reactions. Pyrophosphatase (PPase) hydrolyzes one PPi molecule into two Pi

molecules. Thus, continuously removing PPi from the cytoplasm. Therefore, according to principles of chemical equilibrium, expressing exogenous PPase will shift the equilibrium position of the reactions in the direction of sucrose synthesis. Moreover, exogenous PPase will provide adequate exchanger-Pi for delivering chloroplastic triose phosphate into the cytoplasm.

[0009] To increase the partitioning ratio of carbohydrate assimilate into sucrose, an *E.coli* PPase gene has been cloned into an expression cassette downstream of a 35S promoter, and used to transform potato and tobacco plants. Transcriptional products of this PPase gene are found by Northern blots in both transgenic plants. In leaves of both transgenic plants the ratio of soluble sugars to starch is increased 3-4-fold over wild type. Transgenic tobacco plants show much higher levels of glucose (up to 68-fold), fructose (up to 24-fold), sucrose (up to 12-fold), and starch (up to 8-fold) than non-transgenic plants. Transgenic potato shows a change in assimilate partitioning due to a 2-fold increase in sucrose and a reduction in starch content (Sonnewald U. (1992) *Expression of E. coli inorganic pyrophosphatase in transgenic plants alters photoassimilate partitioning,* Plant J 2:571-581).

[0010] However, prior art transgenic plants exhibit stunted growth, shortened internode distances, and decreased PPi content. Moreover, the soluble sugar content of old source leaves of prior art transgenic plants is 100-fold higher than that of wild type leaves. It is speculated that PPase expression controlled by a constitutive 35S promoter stunts the growth of transgenic plants. PPase expression in vascular bundles of transgenic plants would lead to decreased PPi content, which in turn blocks the loading of sucrose in phloem as PPi is necessary for long distance transporation of sucrose, thus a large amount of sucroses accumulate in the leaves (Sonnewald U. (1992); Lerchl J, Geigenberger P, Stitt M, Sonnewald U (1995) *Impaired photoassimilate partitioning caused by phloem-specific removal of pyrophosphate can be complemented by a phloem-specific cytosolic yeast-derived invertase in transgenic plants*, Plant Cell 7: 259-270).

[0011] Not withstanding the state of the prior art, there continues to be a need for transgenic plants expressing PPase enzymes localized to mesophyll tissues.

SUMMARY OF THE INVENTION

[0012] Various aspects and embodiments of the present invention are addressed to unmet needs in the prior art.

[0013] There is provided a mesophyll-specific, pyrophosphatase expression cassette. The inventive expression cassette comprises a mesophyll-specific expression promoter, a pyrophosphatase gene, and, a terminator. The promoter, pyrophosphatase gene, and terminator may be operably-linked. The promoter, pyrophosphatase gene, and terminator are arranged in seriatem.

[0014] The inventive expression cassette contains a DNA sequence which functions as a mesophyll-specific promoter. The promoter may be a cyFBPase promoter. The promoter comprises the nucleotide bases numbered from 37,511,734 to 37,512,949 from the 5'-end of the DNA sequence having Genebank Number NC_008394.1.

[0015] According to an aspect, the inventive expression cassette contains a DNA sequence which functions as a terminator. The terminator is an OCS terminator. According to a further aspect, the terminator comprises the nucleotide bases numbered from 1403 to 1629 from the 5'-end of the DNA sequence having Genebank Number V00088.

[0016] The inventive expression cassette contains a DNA sequence which encodes a pyrophosphatase enzyme. According to an aspect, the pyrophosphatase gene comprises nucleotide bases 1 to 531 from the 5'-end of the DNA sequence having Genebank Number ABE10235.

[0017] The inventive expression cassette may contain a electable marker gene.

[0018] There is provided a mesophyll-specific, pyrophosphatase expression vector. pect, The inventive expression vector comprises a plasmid having inserted therein a mesophyll-specific, pyrophosphatase expression cassette. The mesophyll-specific recombinant expression vector is a binary expression vector. The plasmid is pCAMBIA 1300. The mesophyll-specific recombinant expression vector further comprises a selectable marker gene.

[0019] There is provided a transgenic cell comprising a mesophyll-specific, pyrophosphatase expression cassette. The cell may be a bacterium or a plant. According to an aspect, the transgenic cell is a plant cell. The transgenic plant cell is a monocotyledon. The transgenic plant cell is a Gramineae.

[0020] There is provided a method for improving plant yield. The method includes transforming a plant with a mesophyll-specific, pyrophosphatase expression vector. The vector comprises an expression cassette which comprises a mesophyll-specific expression promoter; a pyrophosphatase gene; and, a terminator. The expression cassette is arranged such that the promoter, pyrophosphatase gene, and terminator are operably-linked. Further, the promoter is a cyFBPase promoter comprising bases 37,511,734 to 37,512,949 from the 5'-end of Genebank Number NC_008394.1. The terminator is an OCS terminator comprising bases 1403 to 1629 from the 5'-end of Genebank Number V00088. The pyrophosphatase gene comprises bases 1 to 531 from the 5'-end of Genebank Number ABE10235.

[0021] The method includes a mesophyll-specific, pyrophosphatase expression vector. Further, the inventive expression vector comprises a plasmid having inserted therein a mesophyll-specific, pyrophosphatase expression cassette. Further, the mesophyll-specific recombinant expression vector is a binary expression vector. Further, the plasmid is pCAMBIA 1300. Still further, the mesophyll-specific recombinant expression vector further comprises a selectable marker gene.

[0022] The method for improving plant yield further includes screening for plants having an enhanced level of pyrophosphatase. In an aspect, further the method includes screening for enhanced sucrose filling (sink) activity. The method further includes screening for enhanced (number, size, or weight) sink organs.

[0023] There is provided a transformed plant. There is provided a stably-transformed plant. The transformed plant includes a mesophyll-specific, pyrophosphatase expression vector. The vector comprises an expression cassette which comprises a mesophyll-specific expression promoter; a pyrophosphatase gene; and, a terminator. The expression cassette is arranged such that the promoter, pyrophosphatase gene, and terminator are operably-linked. Further, the promoter is a cyFBPase promoter comprising bases 37,511,734 to 37,512,949 from the 5'-end of

Genebank Number NC_008394.1. The terminator is an OCS terminator comprising bases 1403 to 1629 from the 5'-end of Genebank Number V00088. The pyrophosphatase gene comprises bases 1 to 531 from the 5'-end of Genebank Number ABE10235.

[0024] Further, the plant is a monocot. The plant is a Gramineae. The plant is a crop plant. The plant may be rice, wheat, barley or corn.

[0025] Other aspects and advantages of the present invention will become readily apparent by those skilled in the art from the following detailed description, wherein it is shown and described varoius embodiments of the invention, simply by way of illustration of the best mode contemplated of carrying out the invention. As will be realized the invention is capable of other and different embodiments, and its several details are capable of modifications in various obvious respects, without departing from the invention. Accordingly, the description is to be regarded as illustrative in nature and not as restrictive.

BRIEF DESCRIPTION OF DRAWINGS

[0026] The invention is best understood from the following detailed description when read in connection with the accompanying drawing. It is emphasized that, according to common practice, the various features of the drawing are not to scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawing are the following figures:

[0027] Figure 1 Proposed scheme for carbon partitioning in source leaves and the ectopic expression of PPase in transgenic rice. (a) Proposed scheme for the effects of inorganic pyrophosphatase (PPase) on carbon partitioning in source leaves. Triose-P (triose-phosphate), Fru-1,6-P2 (fructose-1,6-bisphosphate), Fru-6-P (fructose-6-phosphate), PPi (inorganic pyrophosphate), Pi (inorganic phosphate), Glu-6-P (glucose-6-phosphate), Glu-1-P (glucose-1-phosphate), UDP-Glu (UDP-glucose), Suc-6-P (sucrose-6-phosphate), FBPase (fructose-1,6-bisphosphatase), PFK (6-phosphofructokinase), PFP (fructose-6-phosphate-1-phosphotransferase), UGPase (UDP-glucose pyrophosphorylase), SPS (sucrose phosphate synthase), SPP (sucrose phosphate phosphatase); (b) PPase activities in the mature leaves of the rice plants. The results are shown as mean ± s.e.m. (n = 7); (c) Pyrophosphate (PPi) content in

mature source leaves. The results are shown as mean ± s.e.m. (n = 8). Abbreviations: FW, fresh weight; PPi (inorganic pyrophosphate); WT, wild type; 29, 34, 37, three transgenic lines. **, P<0.01; ***P<0.001 according to Student's t-test;

[0028] Figure 2 is a schematic diagram of plasmid FBP: PPase;

[0029] Figure 3 shows PCR results of partial FBP: PPase-transgenic rice lines with positive resistance screening results;

[0030] Figure 4 shows the test results of Western (a), PPase activity (b), and PPi content (c) for FBP: PPase-transgenic rice;

[0031] Figure 5 shows the result of sucrose and starch contents, and isotopic tracing in leaves of FBP: PPase-transgenic rice capable of expressing PPase.

[0032] Figure 6 shows diurnal variation of sucrose and starch contents in FBP: PPase-transgenic rice capable of expressing PPase;

[0033] Figure 7 shows metabolite analysis of a FBP: PPase-transgenic rice;

[0034] Figure 8 shows photosynthesis variations of a FBP: PPase-transgenic rice capable of expressing PPase; and,

[0035] Figure 9 shows increased biomass was found in PPase-expressing plants. (**a-b**), the photos were taken 60 days after seeding. (**c**) The photo was taken after harvesting plants. Abbreviations: WT, wild type; 29, 34 and 37, three independent transgenic lines.

[0036] It is to be noted, however, that the appended drawings illustrate only typical embodiments of this invention and are therefore not to be considered limiting of its scope, for the invention may admit to other equally effective embodiments.

DETAILED DESCRIPTION OF AN EXEMPLARY EMBODIMENT

[0037] In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

[0038] The term "gene" is used broadly to refer to any segment of nucleic acid associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. For example, "gene" refers to a nucleic acid fragment that expresses mRNA or functional RNA, or encodes a specific protein, and which includes regulatory sequences. Genes also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins. A gene may also comprise other 5' and 3' untranslated sequences and termination sequences. Further elements that may be present are, for example, introns. Genes can be obtained from a variety of sources, including by cloning from a source of interest or by synthesis using a known or predicted sequence, and may include sequences designed to have desired parameters.

[0039] Associated With/Operatively Linked: Refers to two DNA sequences that are related physically or functionally. For example, a promoter or regulatory DNA sequence is said to be "associated with" a DNA sequence that codes for an RNA or a protein if the two sequences are operatively linked, or situated such that the regulator DNA sequence will affect the expression level of the coding or structural DNA sequence.

[0040] Coding Sequence: a nucleic acid sequence that is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense RNA or antisense RNA. Preferably the RNA is then translated in an organism to produce a protein.

[0041] Expression: refers to the transcription and/or translation of an endogenous gene or a transgene in plants. In the case of antisense constructs, for example, expression may refer to the transcription of the antisense DNA only.

[0042] Expression Cassette: A nucleic acid sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the

expression cassette is heterologous with respect to the host, i.e., the particular nucleic acid sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, such as a plant, the promoter can also be specific to a particular tissue, or organ, or stage of development.

[0043] Heterologous DNA Sequence: The terms "heterologous DNA sequence", "exogenous DNA segment" or "heterologous nucleic acid," as used herein, each refer to a sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified through, for example, the use of DNA shuffling. The terms also includes non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

[0044] The products of photosynthesis are called “assimilates.”

[0045] Most of the interior of the leaf between the upper and lower layers of epidermis is a parenchyma (ground tissue) or chlorenchyma tissue called the mesophyll (Greek for "middle leaf"). This assimilation tissue is the primary location of photosynthesis in the plant.

[0046] Isolated: In the context of the present invention, an isolated nucleic acid molecule or an isolated enzyme is a nucleic acid molecule or enzyme that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated nucleic acid molecule or enzyme may exist in a purified form or may exist in a non-native environment such as, for example, a recombinant host cell.

[0047] Plant: Any whole plant.

[0048] Plant Cell: Structural and physiological unit of a plant, comprising a protoplast and a cell wall. The plant cell may be in form of an isolated single cell or a cultured cell, or as a part of higher organized unit such as, for example, a plant tissue, a plant organ, or a whole plant.

[0049] Plant Cell Culture: Cultures of plant units such as, for example, protoplasts, cell culture cells, cells in plant tissues, pollen, pollen tubes, ovules, embryo sacs, zygotes and embryos at various stages of development.

[0050] Plant Material: Refers to leaves, stems, roots, flowers or flower parts, fruits, pollen, egg cells, zygotes, seeds, cuttings, cell or tissue cultures, or any other part or product of a plant.

[0051] Plant Organ: A distinct and visibly structured and differentiated part of a plant such as a root, stem, leaf, flower bud, or embryo.

[0052] Plant tissue: A group of plant cells organized into a structural and functional unit. Any tissue of a plant in planta or in culture is included. This term includes, but is not limited to, whole plants, plant organs, plant seeds, tissue culture and any groups of plant cells organized into structural and/or functional units. The use of this term in conjunction with, or in the absence of, any specific type of plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue.

[0053] Promoter" refers to a nucleotide sequence, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. "Promoter" includes a minimal promoter that is a short DNA sequence comprised of a TATA box and other sequences that serve to specify the site of transcription initiation, to which regulatory elements are added for control of expression. "Promoter" also refers to a nucleotide sequence that includes a minimal promoter plus regulatory elements that is capable of controlling the expression of a coding sequence or functional RNA. This type of promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue specificity of a promoter. It is capable of operating in both orientations (normal or flipped), and

is capable of functioning even when moved either upstream or downstream from the promoter. Both enhancers and other upstream promoter elements bind sequence-specific DNA-binding proteins that mediate their effects. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even be comprised of synthetic DNA segments. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions.

[0054] The "initiation site" is the position surrounding the first nucleotide that is part of the transcribed sequence, which is also defined as position +1. With respect to this site all other sequences of the gene and its controlling regions are numbered. Downstream sequences (i.e., further protein encoding sequences in the 3' direction) are denominated positive, while upstream sequences (mostly of the controlling regions in the 5' direction) are denominated negative.

[0055] Promoter elements, particularly a TATA element, that are inactive or that have greatly reduced promoter activity in the absence of upstream activation are referred to as "minimal or core promoters." In the presence of a suitable transcription factor, the minimal promoter functions to permit transcription. A "minimal or core promoter" thus consists only of all basal elements needed for transcription initiation, e.g., a TATA box and/or an initiator.

[0056] Minimal Promoter: a promoter element, particularly a TATA element, that is inactive or has greatly reduced promoter activity in the absence of upstream activation. In the presence of a suitable transcription factor, a minimal promoter functions to permit transcription.

[0057] Expression cassette" as used herein means a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA, in the sense or antisense direction. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one which is naturally

occurring but has been obtained in a recombinant form useful for heterologous expression. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, the promoter can also be specific to a particular tissue or organ or stage of development.

[0058] Vector" is defined to include, inter alia, any plasmid, cosmid, phage or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform a prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

[0059] A plant transformation vector comprises one or more DNA vectors for achieving plant transformation. For example, it is a common practice in the art to utilize plant transformation vectors that comprise more than one contiguous DNA segment. These vectors are often referred to in the art as binary vectors.

[0060] Binary vectors as well as vectors with helper plasmids are most often used for *Agrobacterium*-mediated transformation, where the size and complexity of DNA segments needed to achieve efficient transformation is quite large, and it is advantageous to separate functions onto separate DNA molecules. Binary vectors typically contain a plasmid vector that contains the cis-acting sequences required for T-DNA transfer (such as left border and right border), a selectable marker that is engineered to be capable of expression in a plant cell, and a polynucleotide of interest (*i.e.*, a polynucleotide engineered to be capable of expression in a plant cell for which generation of transgenic plants is desired). Also present on this plasmid vector are sequences required for bacterial replication. The cis-acting sequences are arranged in a fashion to allow efficient transfer into plant cells and expression therein. For example, the selectable marker sequence and the sequence of interest are located between the left and right borders. Often a second plasmid vector contains the trans-acting factors that mediate T-DNA transfer from *Agrobacterium* to plant cells. This plasmid often contains the virulence functions (*Vir* genes) that allow infection of plant cells by *Agrobacterium*, and transfer of DNA by cleavage at border sequences and vir-mediated DNA transfer, as in understood in the art (Hellens et al.,

2000). Several types of *Agrobacterium* strains (*e.g.*, LBA4404, GV3101, EHA101, EHA105, *etc.*) can be used for plant transformation. The second plasmid vector is not necessary for introduction of polynucleotides into plants by other methods such as microprojection, microinjection, electroporation, polyethylene glycol, *etc.*

[0061] Specifically included are shuttle vectors by which is meant a DNA vehicle capable, naturally or by design, of replication in two different host organisms, which may be selected from actinomycetes and related species, bacteria and eukaryotic (e.g. higher plant, mammalian, yeast or fungal cells).

[0062] Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate promoter or other regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial, or plant cell. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic DNA, this may contain its own promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

[0063] Cloning vectors" typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance, hygromycin resistance or ampicillin resistance.

[0064] Constitutive expression" refers to expression using a constitutive or regulated promoter. "Conditional" and "regulated expression" refer to expression controlled by a regulated promoter.

[0065] Constitutive promoter" refers to a promoter that is able to express the open reading frame (ORF) that it controls in all or nearly all of the plant tissues during all or nearly all developmental stages of the plant. Each of the transcription-activating elements do not exhibit an absolute tissue-specificity, but mediate transcriptional activation in most plant parts at a level of .gtoreq.1% of the level reached in the part of the plant in which transcription is most active.

[0066] Regulated promoter" refers to promoters that direct gene expression not constitutively, but in a temporally- and/or spatially-regulated manner, and includes both tissue-specific and inducible promoters. It includes natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences. Different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. New promoters of various types useful in plant cells are constantly being discovered, numerous examples may be found in the compilation by Okamuro et al. (1989). Typical regulated promoters useful in plants include but are not limited to safener-inducible promoters, promoters derived from the tetracycline-inducible system, promoters derived from salicylate-inducible systems, promoters derived from alcohol-inducible systems, promoters derived from glucocorticoid-inducible system, promoters derived from pathogen-inducible systems, and promoters derived from ecdysome-inducible systems.

[0067] Tissue-specific promoter" refers to regulated promoters that are not expressed in all plant cells but only in one or more cell types in specific organs (such as leaves or seeds), specific tissues (such as embryo or cotyledon), or specific cell types (such as leaf parenchyma or seed storage cells). These also include promoters that are temporally regulated, such as in early or late embryogenesis, during fruit ripening in developing seeds or fruit, in fully differentiated leaf, or at the onset of senescence.

[0068] Recombinant DNA molecule: a combination of DNA molecules that are joined together using recombinant DNA technology.

[0069] Regulatory Elements: Sequences involved in controlling the expression of a nucleotide sequence. Regulatory elements comprise a promoter operably linked to the nucleotide sequence of interest and termination signals. They also typically encompass sequences required for proper translation of the nucleotide sequence.

[0070] Selectable marker gene: a gene whose expression in a plant cell gives the cell a selective advantage. The selective advantage possessed by the cells transformed with the selectable marker gene may be due to their ability to grow in the presence of a negative selective agent, such as an antibiotic or a herbicide, compared to the growth of non-transformed cells. The

selective advantage possessed by the transformed cells, compared to non-transformed cells, may also be due to their enhanced or novel capacity to utilize an added compound as a nutrient, growth factor or energy source. Selectable marker gene also refers to a gene or a combination of genes whose expression in a plant cell gives the cell both, a negative and a positive selective advantage.

[0071] Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). Additional selectable markers include phenotypic markers such as β-galactosidase and fluorescent proteins such as green fluorescent protein, cyan florescent protein, and yellow florescent protein.

[0072] Significant Increase: an increase in enzymatic activity that is larger than the margin of error inherent in the measurement technique, preferably an increase by about 2-fold or greater of the activity of the wild-type enzyme in the presence of the inhibitor, more preferably an increase by about 5-fold or greater, and most preferably an increase by about 10-fold or greater.

[0073] Two nucleic acids are "recombined" when sequences from each of the two nucleic acids are combined in a progeny nucleic acid. Two sequences are "directly" recombined when both of the nucleic acids are substrates for recombination. Two sequences are "indirectly recombined" when the sequences are recombined using an intermediate such as a cross-over oligonucleotide. For indirect recombination, no more than one of the sequences is an actual substrate for recombination, and in some cases, neither sequence is a substrate for recombination.

[0074] Transformation: a process for introducing heterologous DNA into a host cell or organism.

[0075] "Transformed," "transgenic," and "recombinant" refer to a host organism such as a bacterium or a plant into which a heterologous nucleic acid molecule has been introduced. The nucleic acid molecule can be stably integrated into the genome of the host or the nucleic acid molecule can also be present as an extrachromosomal molecule. Such an extrachromosomal

molecule can be auto-replicating. Transformed cells, tissues, or plants are understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof. A "non-transformed," "non-transgenic," or "non-recombinant" host refers to a wild-type organism, e.g., a bacterium or plant, which does not contain the heterologous nucleic acid molecule.

[0076] In an embodiment, an inorganic pyrophosphatase (PPase) gene is expressed in plant mesophyll cells under the regulation of a mesophyll-specific expression promoter. PPase activity, by continuously removing PPi from the cytoplasm shifts the equilibrium of sucrose-based reactions in the direction of sucrose synthesis. Moreover, increased PPase expression in mesophyll (source) cells, alters carbohydrate partitioning towards sink organs. and provides adequate exchanger-Pi for delivering triose phosphate into cytoplasm. In addition, mesophyll-specific expression of sucrose prevents the loading of sucrose in phloem from being blocked due to PPi content reduction in phloem, particularly in companion cells, then plant yield is improved.

[0077] The inventive method has been experimentally-demonstrated to significantly improve yields of transgenic plants, especially of transgenic grain plants. It has been showed by experiment, that rice (*Indica* variety 6547 and *Japonica* variety 8706), transformed by expression cassettes of the present invention, express PPase localized to mesophyll cells. Moreover, carbohydrate partitioning in transgenic plants is improved and the yields are increased by up to 50 %.

[0078] The methods used in the examples below are conventional, unless otherwise indicated. The percentages indicated are percentages by weight, unless otherwise indicated.

[0079] According to the invention, a promoter whose controlled expression is limited to plant mesophyll cells (cyFBPase promoter) and a PPase gene are operably-linked in an expression cassette. Expression constructs, cassettes and vectors, containing an operably-linked cyFBPase promoter and a PPase gene are used to transform plant cells. PPase genes direct the expression of PPase functional enzyme in transformed rice under the regulation of a mesophyll-specific expression promoter. Continuously removing PPi in cytoplasm by expressing PPase induces the synthetic equilibrium in the direction of sucrose synthesis, and provides adequate

exchanger-Pi for delivering triose phosphate into cytoplasm. As a result, carbohydrate assimilate partitioning in plants is altered and improved plant yields are obtained.

[0080] As an embodiment of the present invention, a transgenic rice with improved yield is constructed, and carbohydrate assimilates partitioning and content thereof are examined. various method embodiments are also disclosed.

[0081] Example 1. Construction of PPase-expressing transgenic rice.

[0082] *1.1 Construction of a recombinant vector capable of expressing PPase (FBP: PPase vector).* Plasmid pCambia 1391Z containing a cyFBPase promoter (Cambia, Canberra, Australia) is digested by restriction enzyme *Eco*R1 to obtain a fragment of 1229 bp, i.e. cyFBPase promoter (nucleotide acids 37511734-37512949 from 5' end of Genebank Number NC-008394.1). The resultant cyFBPase promoter is inserted into the *Eco*R1 site of PCAMBIA 1300 to obtain recombinant vectors. The recombinant vectors are validated by enzyme digestion and sequencing, and the recombinant vector pCAMBIA1300 (Cambia, Australia) containing cyFBPase promoter fragment is designated pCA-FBP.

[0083] A PPase gene fragment of about 530 DNA bp was obtained by PCR amplification using *E.coli* (*Escherichia coli* XL1-blue strain, Labpil Biotech Ltd., Beijing, CN) genome as a template and a forward, 5' primer, F1: 5′ GGATCCATGAGCTTACTCAACGTCCCTGCGGGT 3′ (SEQ ID NO: 1) and a reverse 3' primer R1: 5′ GGATCCTTATTTATTCTTGGCGCGCTCGAA 3′ (SEQ ID NO: 2). The PPase gene fragment was sequenced and shown to comprise nucleotide acids 1-531 from the 5' end of Genebank Number ABE10235. The amplified PPase gene fragment was digested with *Bam*H1 and inserted between the *Bam*H1 sites of pUC19 (Labpil Biotech Ltd., Beijing, CN) to obtain recombinant vectors. The recombinant vectors are validated by enzyme digestion and sequencing, and the recombinant vector containing PPase gene is designated as pUC-PPase.

[0084] Vector pUC-PPase is digested with *Bam*HI, the PPase gene fragment (containing the sequence of nucleotide acids 1-531 from 5' end of Genebank Number ABE10235) is recovered, and is inserted into *Bam*H1 sites of pCA-FBP forward, i.e. behind the cyFBpase promoter of pCA-FBP, to obtain recombinant vectors. The recombinant vectors are validated by enzyme

digestion and sequencing, and the pCAMBIA1300 recombinant vector correctly containing cyFBpase promoter and forward PPase gene fragment in series is designated as pCA-FBP-PPase.

[0085] Terminator OCS (227 bp DNA) was obtained by PCR amplification using *Agrobacterium tumefaciens* AGL1 (American Type Culture Collection) as a template, a (forward) 5' primer F2: 5′ CAGGGCTCTCAATGGAGTTTGAA 3′ (SEQ ID NO: 3), and a (reverse) 3' primer R2: 5′ CAATCAGTAAATTGAAC GGAGA 3′ (SEQ ID NO: 4). The OCS terminator fragment was sequenced and shown to comprise base pairs 1403-1629 from 5' end of Genebank Number V00088.

[0086] The OCS terminator fragment was digested with restriction enzymes *Sal*I and *Hin*dIII and inserted between the *Sal*I site and *Hin*dIII sites of pCA-FBP-PPase to obtain recombinant vectors. The recombinant vectors are validated by enzyme digestion and sequencing, and the recombinant vector correctly containing cyFBpase promoter, forward PPase gene fragment, and terminator OCS, in series, is designated FBP:PPase (schematic diagram as shown in Figure 2). In Figure 2, 35S-pro is 35S CaMV promoter, FBP-pro is cyFBPase promoter, t35 is CaMV 35S terminator, Hygromycin is Hygromycin-resistance selective gene, and tOCS is OCS terminator.

[0087] 1.2 Production and identification of PPase-expressing transgenic rice.

[0088] *1.2.1. Production of PPase transgenic rice (FBP: PPase transgenic rice).* Callus of Japonica rice, variety 8706 (The Institute of Genetics and Developmental Biology of the Chinese Academy of Sciences) is transformed with vector FBP: PPase vector using *Agrobacterium tumefaciens* AGL1 (purchase from *ATCC*) yielding a T_0 generation of FBP: PPase-transgenic rice lines with positive resistance screening result. Transformation methods are described in Yi Zi-Li,CAO Shou-Yun, et al. (2001). *Improvement of Transformation Frequency of Rice Mediated by Agrobacterium*, Acta Genetica Sinica 28 (4): 352-358.

[0089] 1.2.2. *PCR identification of PPase-transgenic rice (FBP: PPase-transgenic rice).* PCR amplification is performed using genome DNA purified from FBP: PPase-transgenic rice lines obtained above as a template and primers specific for PPase gene encoding region (upstream primer F3: ATGAGCTTACTCAACGTCCCTGCGGGT, SEQ ID NO: 5) and downstream primer R3: TTATTTATTCTTGGCGCGCTCGAA, SEQ ID NO: 6) as primers and

wild-type rice (8706) as a control. PCR amplification of eleven strains of FBP: PPase transgenic rice lines demonstrated expected bands of about 500 bp. Whereas, no amplified band is observed for the non-transgenic, negative control. And it has been shown by sequencing that the amplified fragments are PPase gene fragments. Thus 11 FBP: PPase-transgenic rice lines are obtained. PCR results of partial FBP: PPase-transgenic rice lines with positive resistance screening results are shown as Figure 3. In figure 2, lane 1 is a MW Standard Marker; lane 2 is non-transgenic rice negative control; and lanes 3-13 are amplification results of FBP: PPase-transgenic rice lines with positive resistance screening results.

[0090] 1.2.3. *Screening for PPase gene-transgenic rice capable of expressing PPase (FBP: PPase-transgenic rice).* Three FBP: PPase-transgenic homozygous lines, lines 27, 34, and 73, are obtained by Hygromycin screening and identification in descendants. Total protein (30 μg) from fully-expanded mature leaves of non-transgenic rice (rice 8706, negative control, WT) and FBP: PPase-transgenic rice lines 27, 34, and 73 were heat denatured, electrophoreses on SDS-PAGE gels, transferred to nitrocellulose membranes, and Western blotted using PPase-specific antibody (Preparation method of antibody is to inject a rabbit with *E.coli* PPase protein expressed by E.coli three times (once every 10 days for a month), and collect serum from the rabit). Western blots showed no bands for non-transgenic negative control plants (WT) and there is a 32kD specific band as expected for the FBP:PPase transgenic rice lines 27, 34, and 37 (as shown in Figure 4a), indicating that three FBP:PPase transgenic rice lines expressing exogenous PPase gene are obtained. Seeds of T_0 generation of the FBP:PPase transgenic rice plants and the plants grown from the seeds are T_1 generation of the FBP:PPase transgenic rice plants, and successively seeds of T_1 generation and the plants grown from the seeds are T_2 generation.

[0091] 2. Analysis of PPase activity in leaves of FBP:PPase transgenic rice.

[0092] PPase activities in non-transgenic rice (rice 8706, negative control, WT) and the three FBP:PPase transgenic rice lines 27, 34, and 37 obtained above are examined, wherein the methods and steps are described in Sonnewald, U. (1992) *Expression of E.coli inorganic pyrophosphatase in transgenic plants alters photoassimilate partitioning*. Plant J. 2, 571-581. As shown in figure 4(b), the result indicates that PPase activities in the FBP:PPase transgenic rice lines capable of expressing PPase obtained in step 1 are 3-5 times higher than that of non-

transgenic rice control. These results show that FBP:PPase transgenic rice express higher levels of PPase activity.

[0093] *Inorganic pyrophosphate (PPi) content of FBP:PPase transgenic rice.* The PPi content of FBP:PPase transgenic rice lines 27, 34, and 37 and of non-transgenic rice (rice 8706, negative control, WT) were determined by the method of Sonnewald, U. (1992) *Expression of E. coli inorganic pyrophosphates in transgenic plants alters photoassimilate partitioning*, Plant J. 2, 571-581. PPi is extracted and loaded on a 5µm Hamilton PRP-X100 (100×4.1 mm) anion exchange column coupled to a 25×2.3 mm guard (Hamilton Inc., USA) with 1.0 ml/min 60 mM ammonia/100 mM formic acid as mobile phase and analyzed on a HPLC 10Avp system (Shimadzu, Japan) and a Corona Charged Aerosol Detector (ESA Inc., USA). Sodium pyrophosphate is used as a standard. The PPi content of each transgenic plant line decreased significantly compared to non-transgenic control (WT) (Figure 4c).

[0094] 3. Carbohydrate analysis of FBP:PPase transgenic rice leaves.

[0095] FBP:PPase transgenic rice (lines 29, 34, and 37) were compared for carbohydrate content against wild-type, non-transgenic rice (rice 8706) by the method of Geigenberger, P. *et al.* (1998) *Overexpression of pyrophosphatase leads to increased sucrose degradation and starch synthesis, increased activities of enzymes for sucrose-starch interconversions, and increased levels of nucleotides in growing potato tubers*. Planta 205, 428-437. Sucrose and starch were extracted from fully-expanded, mature leaf. Figure 5a shows that sucrose content of each of the three FBP:PPase transgenic rice lines does not significant differ from the wild-type control, but starch contents are 4-5 times lower than that of the non-transgenic control (Figure 5a).

[0096] To prove that photosynthetically-fixed CO_2 flowed in the direction of sucrose synthesis, $^{14}CO_2$ tracing experiments were performed on leaves of three FBP:PPase transgenic rice lines (lines 29, 34 and 37) and compared to control, non-transgenic rice (rice 8706). Leaf discs are cut directly from fully-expanded flag leaves from control and three FBP:PPase transgenic rice lines (lines 29, 34 and 37) from field-grown plants (60-days after sowing) using a 1 cm cork borer. Discs were quickly positioned on a wet sponge in a specially constructedair-tight, humidity-controlled organic glass chamber. A petri dish containing 2 ml of 10 mM MES

(2-(N-morpholino) ethane sulphonic acid) adjusted to pH 6.5 with KOH) and 40 μCi of $NaH^{14}CO_3$ was placed in the chamber to serve as a source of radiolabeled carbon. Radiocarbon was released by adding 2-3 ml of 10 % 1 mol/L hydrochloric acid to the petri dish. The chamber was immediately closed and sealed following the addition. A 250 μ Einstein light source was placed 60 cm above the chamber. After a 1-hour incubation, leaf discs were collected, extracted with 80 % (v/v) ethanol at 80°C for 1 hour; and the radioactivity of the soluble and insoluble fractions was quantified by liquid scintillation counting. Figure 5b shows a significant reduction in the incorporation of newly fixed ^{14}C into the insoluble fraction and a significant increase in the incorporation into soluble fraction in all three transgenic lines, compared to the non-transgenic control. These results show that the significant decrease in the starch content of FBP:PPase transgenic rice leaf results from a diversion of newly-fixed CO_2 from starch synthesis, within the leaf, to sucrose synthesis pathway. Moreover, most of the newly-synthesized sucrose is delivered to sink organs through plant vascular tissues and the sucrose does not accumulate in source leaves. Thus it can be seen that mesophyll-specific expression of PPase in FBP:PPase transgenic rices capable of expressing PPase alters the carbohydrate partitioning.

[0097] 4. Diurnal variation of carbohydrate assimilate partitioning in FBP: PPase-transgenic rice leaf.

[0098] At six time-points during the day (10:00, 14:00, 18:00, 22:00, 2:00, and 6:00), the variation in the soluble sugar and starch content of leaves was sampled from the FBP:PPase transgenic (T_1 generation plants of lines 29, 34, and 37, 5 plants per line) and wild-type rice (8706, 5 plants) according to the method of Geigenberger, P. *et al.* (1998). As shown in Figure 6, the diurnal variation of sucrose is regular. The sucrose content of FBP:PPase transgenic rice is relatively low from 2:00 to 6:00, begins to rise at 10:00, reaches the maximum by 14:00, and then declines. Although at night, the starch content in FBP:PPase transgenic rice is lower than that of non-transgenic control, it is enough for plant to maintain normal nocturnal activity. In Figure 6, WT is the average measurement of 5 individual plants of non-transgenic control, and 29, 34, and 37 are the average measurement result of 5 individual plants from the three FBP:PPase transgenic rice lines 29, 34, and 37 respectively.

[0099] 5. Metabolite analysis of FBP:PPase transgenic rice.

[00100] Metabolite analysis wwere determined for FBP:PPase transgenic rice (T_1 generation, lines 29, 34, and 37, 5-7 plants per line) and wild-type, non-transgenic control rice (rice 8706, 5-7 plants) according to the method of Geigenberger, P. *et al* (1998). As shown in Figure 7, the fructose-6-phosphate and glycerate-3-phosphate content of FBP:PPase transgenic rice does not differ significantly from control. Glucose-6-phosphate content increases 83 % in transgenic line 29, 26% and 12% in lines 34 and 37 respectively, compared to control. The UDP-glucose contents are much higher in the three transgenic lines (29, 3-fold; 34, 2-fold; and 37, 1.18-fold) than those in non-transgenic control. UDP-glucose is one of the reactants for sucrose synthesis and is found only in cytoplasm, so its significant increase in content indicates that metabolic reaction does move towards the direction of sucrose synthesis. In Figure 7, WT is the average measurement of non-transgenic control (5-7 plants), and 29, 34, and 37 are the average measurement of 5-7 individual plants from FBP:PPase transgenic rice lines 29, 34 and 37.

[00101] 6. Photosynthesis analysis of FBP:PPase transgenic rice.

[00102] Too rapid export of plant photosynthesis product from leaves will lead to reduction of Calvin cycle intermediates, and therefore, a reduction in photosynthesis rate. To study the variation in photosynthesis rate in FBP:PPase transgenic rice, we examine the photosynthetic rate of FBP:PPase transgenic rice under various light intensities (0 - 2000 $\mu mol \cdot m^{-2}s^{-1}$). Net CO_2 uptake rate of rice is measured by examining photosynthesis of flag leaves in the early filling period. Light-response curve is measured with a portable photosynthesis system LI-6400 (Li-cor, Lincoln, NE, USA). The parameters are set as follows: CO_2 flow rate is 400 $\mu mol \cdot sec^{-1}$, air flow rate is 500 $\mu mol \cdot sec^{-1}$ and temperature is 28°C. A range of light intensities between 0 and 2000 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ are supplied by an LED source attached on the leaf chamber. As shown in Figure 7, the result shows that, compared to non-transgenic control, the net CO_2 assimilate rate of FBP:PPase transgenic rices capable of expressing PPase is significantly increased. In addition, at the light saturation point, photosynthetic rate of the transgenic plant is 20 % higher than non-transgenic control. These observations suggest that mesophyll-specific expression of PPase can enhance the photosynthesis of transgenic rice.

[00103] 7. Yield evaluation of FBP:PPase transgenic rice.

[00104] FBP:PPase transgenic rice (lines 29, 34, and 37) was chosen for small scale field experiment and a non-transgenic line(WT rice 8706) was chosen as control. Sixty plants from each FBP:PPase transgenic and WT control lines were planted. After seeds completely matured, 10 plants were sampled for each line and plant heights, tiller number per plant, fully-filled seed number per panicle, total seed number per panicle, percentage of fully-filled seeds, 1000-seed weight, and plant yields were determined. These data were used to calculate increases in the yields of transgenic lines compared to non-transgenic controls. Yield increase (%) = (transgenic lines – non-transgenic control)/non-transgenic control x 100%. The result shows that, growth of either aboveground part or underground part of FBP:PPase transgenic rice is more vigorous than that of WT. Improvements can be seen for tiller number per plant, fully-filled seed number per panicle, total seed number per panicle, and percentage of fully-filled seeds, compared to WT; but plant height, 1000-seed weight, and the like have no significant changes. Individual plant yield increases by 24-50 % compared to the control.

Table 1 Agronomic traits and yield evaluation of FBP:PPase transgenic rices

Agronomic traits	WT	line 29	line 34	line 37
Plant height(cm)	143.8±2.6	139.8±3.3	126.4±1.1*	138.4±3.5
Tiller number per plant	10.6±0.3	13.0±0.3*	13.6±0.6*	12.2±0.4*
Fully-filled seed number per panicle	216.8±4.3	292.5±2.4**	281.0±2.3**	244.4±3.4**
Total seed number per panicle	275.0±6.3	349.2±2.7**	330.5±4.5**	294.4±3.2*
Percentage of fully-filled seeds (%)	79±0.5	84±0.5*	85±0.8*	83±0.8*
1000-seed weight (g)	28.0±1.0	26.1±0.3	23.9±0.4*	27.5±1.2
Yield (g/plant)	66.0±2.9	99.3±3.5**	91.5±4.1**	82.0±2.7**
Yield Increase (%)	NA	50%	39%	24%

[00105] FBP:PPase transgenic rice (lines 29, 34, and 37) and non-transgenic control (WT) are chosen for small-scale yield experiment in Beijing(2004) and Zhejiang(2005) respectively. In

the large-scale field experiments in Beijing and Zhejiang, all experiments are replicated for 6 times and each plot is about 133.3 m^2. The FBP:PPase transgenic rice lines and WT control (rice 8706) are grown in the field at a distance of 25cm x 15cm. After seeds completely matured, actual yield measurements are performed for the FBP:PPase transgenic rice lines and WT control (rice 8706), and then theory yields thereof and yield increase compared to control are calculated. Theory yield (Kg/Hectare) = Actual yield x 75. Yield increase (%) = (transgenic lines – non-transgenic control)/non-transgenic control x 100%. As shown in Figure 3, the result shows that, yields of three FBP:PPase transgenic rices capable of expressing PPase (lines 29, 34, and 37) in Beijing increase by 42%, 32%, and 24% respectively, compared to those of control; Similar results are found in Zhejiang Province in which the yields increase by 22-47 % (Zhejiang, 2005).

able 2 Large-scale field yield experiment
FBP:PPase transgenic rice

Lines	Beijing		Zhejiang	
	Yield (kg/ha)	% Increase	Yield (kg/ha)	% Increase
WT control	4624.2±134.6	—	4098.5±81.9	-
29	6580.0±187.2	42%	6022.5±191.8	47%
34	6082.5±165.3	32%	5257.5±42.6	28%
37	5721.2±149.3	24%	5022.5±47.7	22%

[00106] Example 2. Creation and yield evaluation of PPase-expressing, transgenic *Indica* rice variety 6547.

[00107] To verify whether specific expression of PPase could also improve yield in various rice cultivars, we also express FBP:PPase in an *Indica* rice variety 6547 through the *Agrobacterium* method and perform field experiment of transgenic plant yield. The FBP: PPase vector is transformed into an *Indica* rice variety 6547 (Yanzhou University) according to the method of example 1, and two FBP:PPase transgenic rice lines capable of expressing PPase (lines 3003 and 3010) are obtained by screening, which are selected for large-scale field plant experiments in 2004 and 2005 respectively. Each experiment is set up in three locations, each location includes two replicates, and each plot is about 133.3 m^2. Plants of all lines including WT control are grown in the field at a distance of 25 cm x 15 cm. After seeds completely matured, the FBP:PPase transgenic rice lines are sampled to measure yield according to the method of step

7 in example 2, and non-transgenic *Indica* rice variety 6547 treated equally is chosen as control. The experiment result shows that, compared to control, line 3003 yield increases 11 % (year 2004) and 18 % (year 2005) ; line 3010 yield increases 8 % (year 2004) and 21 % (year 2005) (Table 3).

Table 3 Large-scale field yield experiment
FBP:PPase transgenic *Indica* rice

Lines	Year 2004		Year 2005	
	Yield (kg/ha)	Yield Increase	Yield (kg/ha)	Yield Increase
Indica 6547	6253.2±214.2	——	5145.9±118.5	——
3003	6946.1±135.3	11%	6069.4±98.4	18%
3010	6766.6±64.4	8%	6208.0±181.9	21%

[00108] Reference is made to the figures to illustrate selected embodiments and modes of carrying out the invention. It is to be understood that the invention is not hereby limited to those aspects depicted in the figures.

[00109] The foregoing description of the invention illustrates and describes the present invention. Additionally, the disclosure shows and describes only exemplary embodiments of the invention but, as mentioned above, it is to be understood that the invention is capable of use in various other combinations, modifications, and environments and is capable of changes or modifications within the scope of the inventive concept as expressed herein, commensurate with the above teachings and/or the skill or knowledge of the relevant art. The embodiments described hereinabove are further intended to explain best modes known of practicing the invention and to enable others skilled in the art to utilize the invention in such, or other, embodiments and with the various modifications required by the particular applications or uses of the invention. Accordingly, the description is not intended to limit the invention to the form disclosed herein. Also, it is intended that the appended claims be construed to include alternative embodiments.

SEQUENCE LISTING

<110> Institute of Genetics and Developmental Biology

Chu, Chengcai

Si, Lizhen

Zhang, Yafang

Tong, Hongning

Wang, Yiqin

Pang, Xuebiao

Hajirezai, Mohanmad

Sonnewald, Uwe

<120> Expression Cassettes and Methods for Increasing Plant Yields

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CLAIMS

We claim:

1. A mesophyll-specific, pyrophosphatase expression cassette comprising:

a mesophyll-specific expression promoter;

a pyrophosphatase gene; and,

a terminator,

wherein said promoter, pyrophosphatase gene, and terminator are operably-linked.

2. The mesophyll-specific, pyrophosphatase expression cassette according to Claim 1, wherein said promoter, pyrophosphatase gene, and terminator are arranged in seriatem.

3. The mesophyll-specific, pyrophosphatase expression cassette according to Claim 1, wherein said promoter is a cyFBPase promoter comprising bases 37,511,734 to 37,512,949 from the 5'-end of Genebank Number NC_008394.1.

4. The mesophyll-specific, pyrophosphatase expression cassette according to Claim 1, wherein said terminator is an OCS terminator comprising bases 1403 to 1629 from the 5'-end of Genebank Number V00088.

5. The mesophyll-specific, pyrophosphatase expression cassette according to Claim 1, wherein said pyrophosphatase gene comprises bases 1 to 531 from the 5'-end of Genebank Number ABE10235.

6. A mesophyll-specific recombinant expression vector comprising:

a plasmid having inserted therein a mesophyll-specific, pyrophosphatase expression cassette further comprising:

a mesophyll-specific expression promoter;

a pyrophosphatase gene; and,

a terminator,

wherein said promoter, pyrophosphatase gene, and terminator are operably-linked.

7. The mesophyll-specific recombinant expression vector according to Claim 6,

wherein said promoter is a cyFBPase promoter comprising bases 37,511,734 to 37,512,949 from the 5'-end of Genebank Number NC_008394.1;

wherein said terminator is an OCS terminator comprising bases 1403 to 1629 from the 5'-end of Genebank Number V00088; and,

wherein said pyrophosphatase gene comprises bases 1 to 531 from the 5'-end of Genebank Number ABE10235.

8. The mesophyll-specific recombinant expression vector according to Claim 6 wherein said expression vector is a binary expression vector.

9. The mesophyll-specific recombinant expression vector according to Claim 6 wherein said plasmid is pCAMBIA 1300.

10. The mesophyll-specific recombinant expression vector according to Claim 6, further comprising a selectable marker gene.

11. A transgenic cell comprising the expression cassette of Claim 1.

12. The transgenic cell according to Claim 11, wherein said cell is a cell selected from the group consisting of a bacterium and a plant.

13. The transgenic cell according to Claim 12, wherein said plant is a monocotyledon.

14. The transgenic cell according to Claim 13, wherein said monocotyledon is a Gramineae.

15. A method for improving plant yield comprising transforming a plant with a mesophyll-specific, pyrophosphatase expression vector, said vector comprising:

an expression cassette further comprising:

a mesophyll-specific expression promoter;

a pyrophosphatase gene; and,

a terminator,

wherein said promoter, pyrophosphatase gene, and terminator are operably-linked.

16. The method for improving plant yield according to Claim 15,

wherein said promoter is a cyFBPase promoter comprising bases 37,511,734 to 37,512,949 from the 5'-end of Genebank Number NC_008394.1;

wherein said terminator is an OCS terminator comprising bases 1403 to 1629 from the 5'-end of Genebank Number V00088; and,

wherein said pyrophosphatase gene comprises bases 1 to 531 from the 5'-end of Genebank Number ABE10235.

17. The method for improving plant yield according to Claim 15, further comprising screening for plants having an enhanced level of pyrophosphatase.

18. The method for improving plant yield according to Claim 15, werein said expression vector further comprises a plasmid operably-linked to said cassette.

19. A method for the preparation of a plant with an increased dry weight of a sink organ comprising:

transforming a plant with a mesophyll-specific recombinant expression vector comprising:

a plasmid having inserted therein a mesophyll-specific, pyrophosphatase expression cassette further comprising:

a mesophyll-specific expression promoter;

a pyrophosphatase gene; and,

a terminator,

wherein said promoter, pyrophosphatase gene, and terminator are operably-linked.

20. The method according to Claim 19, wherein the sink organ is selected from the group consisting of seeds, fruits, roots and tubers.

21. A plant stably transformed with an expression vector according to Claim 19.

22. A plant according to Claim 21, wherein the plant is a crop plant.

23. A plant according to claim 22, wherein the crop plant is selected from the group consisting of tobacco, potato, tomato, sugar beet, soya bean, wheat, barley, corn, oat, rice and sugar cane plants.

24. A plant cell transformed with a mesophyll-specific recombinant expression vector comprising:

a plasmid having inserted therein a mesophyll-specific, pyrophosphatase expression cassette further comprising:

a mesophyll-specific expression promoter;

a pyrophosphatase gene; and,

a terminator,

wherein said promoter, pyrophosphatase gene, and terminator are operably-linked,

wherein said promoter is functionally capable of directing the expression of said pyrophosphatase to a desired location in a plant cell.

25. A plant cell according to claim 24, wherein said desired location is a plant mesophyll cell.

26. A plant cell according to claim 25, wherein the plant cell is obtained from a crop plant.

27. A plant cell according to claim 26, wherein the plant cell is obtained from a monocot.

28. A method for the production of transgenic plants, comprising:

producing an expression cassette having the following sequences:

a mesophyll-specific expression promoter;

a pyrophosphatase gene; and,

a terminator,

wherein said promoter, pyrophosphatase gene, and terminator are operably-linked,

transferring said expression cassette into a plant cell, thereby producing a transformed plant cell; and

regenerating whole, intact transgenic plants from said transformed cell.

Figure 1a

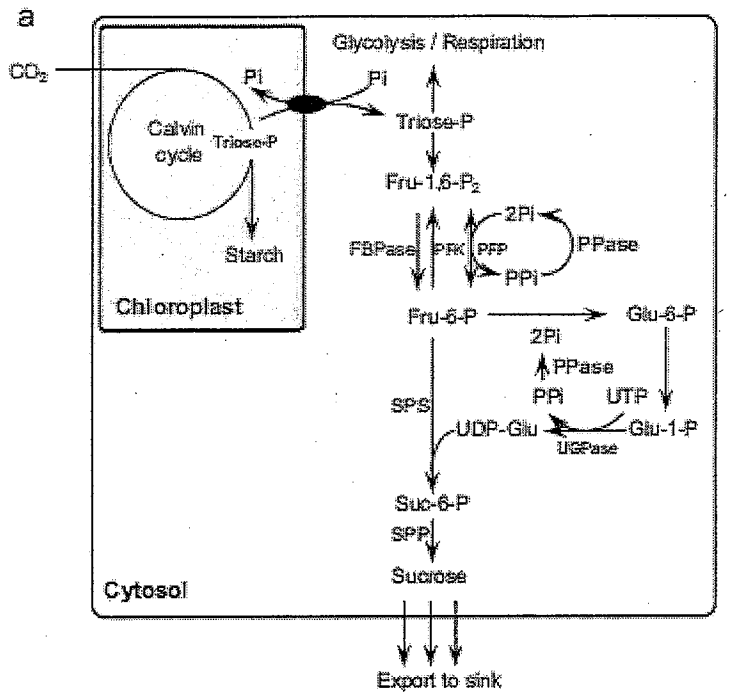

a
CO_2
Pi
Calvin cycle
Triose-P
Starch
Chloroplast
Glycolysis / Respiration
Pi
Triose-P
Fru-1,6-P_2
FBPase
PFK
PFP
2Pi
PPi
PPase
Fru-6-P
Glu-6-P
2Pi
PPase
PPi
UTP
SPS
UDP-Glu
Glu-1-P
UGPase
Suc-6-P
SPP
Sucrose
Cytosol
Export to sink

Figure 1b

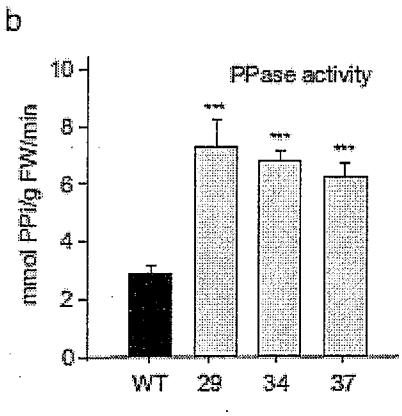


Figure 1c

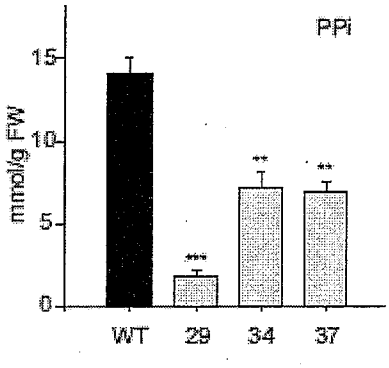


Figure 2

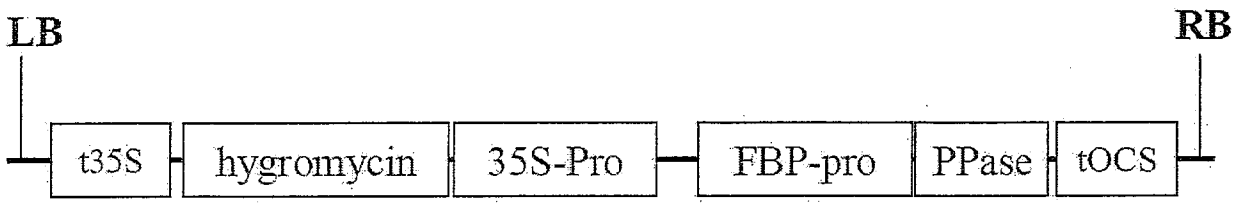


Figure 3

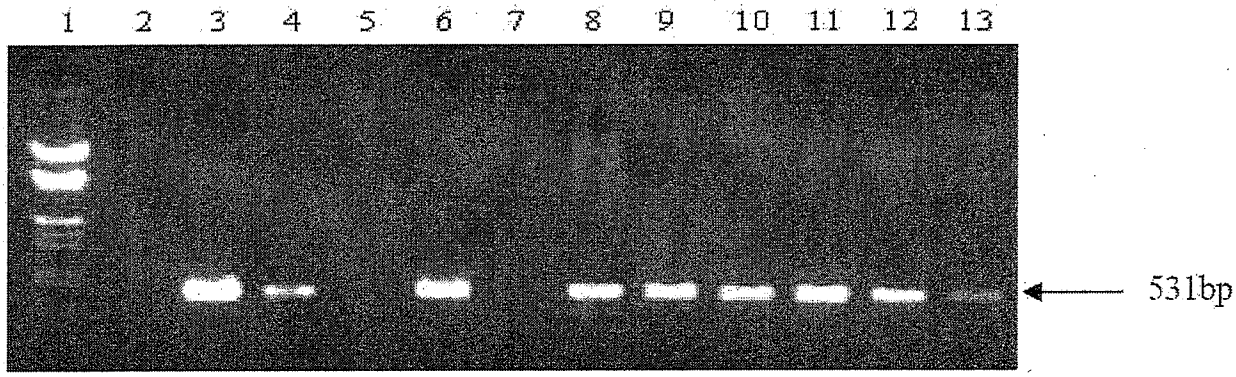


Figure 4

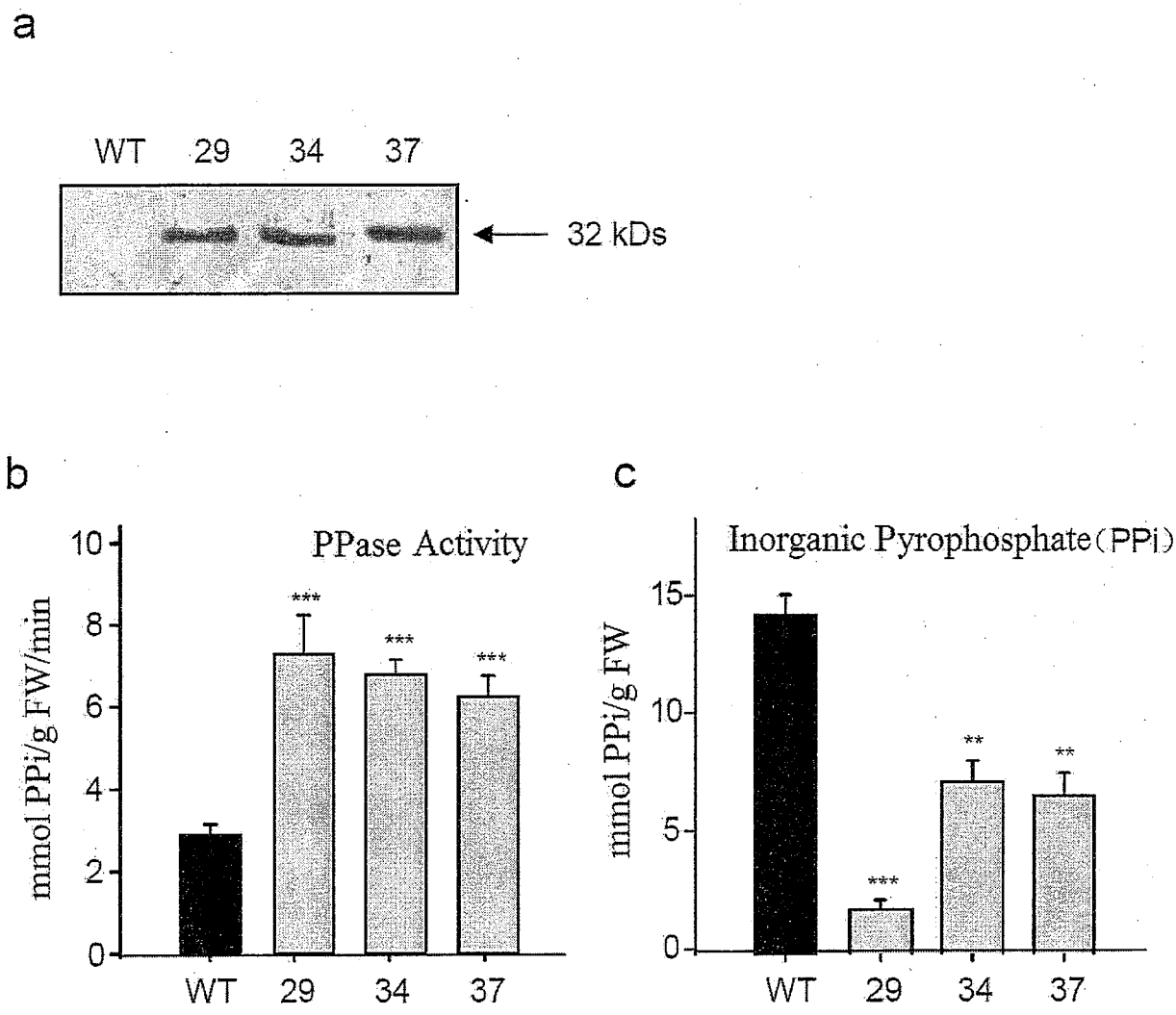

a
WT
29
34
37
32 kDs
b
PPase Activity
mmol PPi/g FW/min
10
8
6
4
2
0
***
***
***
WT
29
34
37
c
Inorganic Pyrophosphate(PPi)
mmol PPi/g FW
15
10
5
0
***
**
**
WT
29
34
37

Figure 5

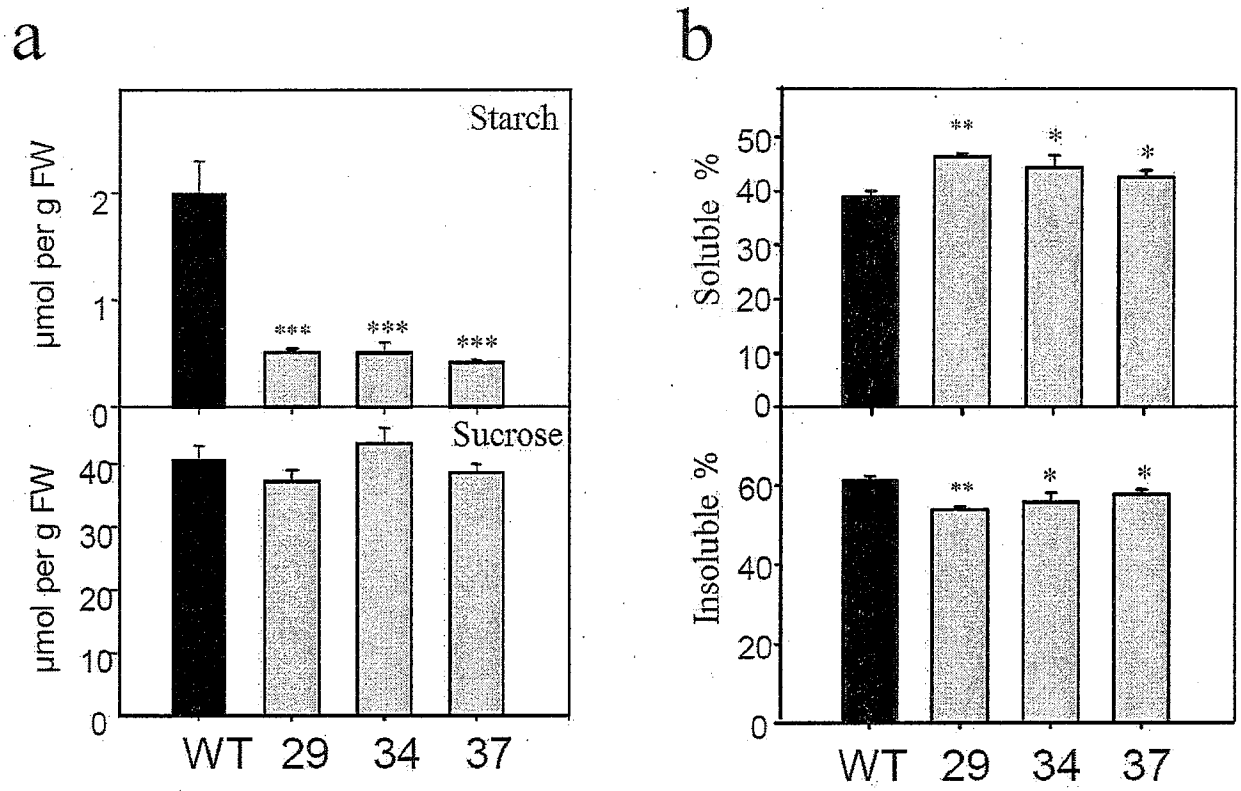

a
Starch
µmol per g FW
2
1
0
***
***
***
Sucrose
µmol per g FW
40
30
20
10
0
WT
29
34
37
b
Soluble %
50
40
30
20
10
0
**
*
*
Insoluble %
60
40
20
0
**
*
*
WT
29
34
37

Figure 6

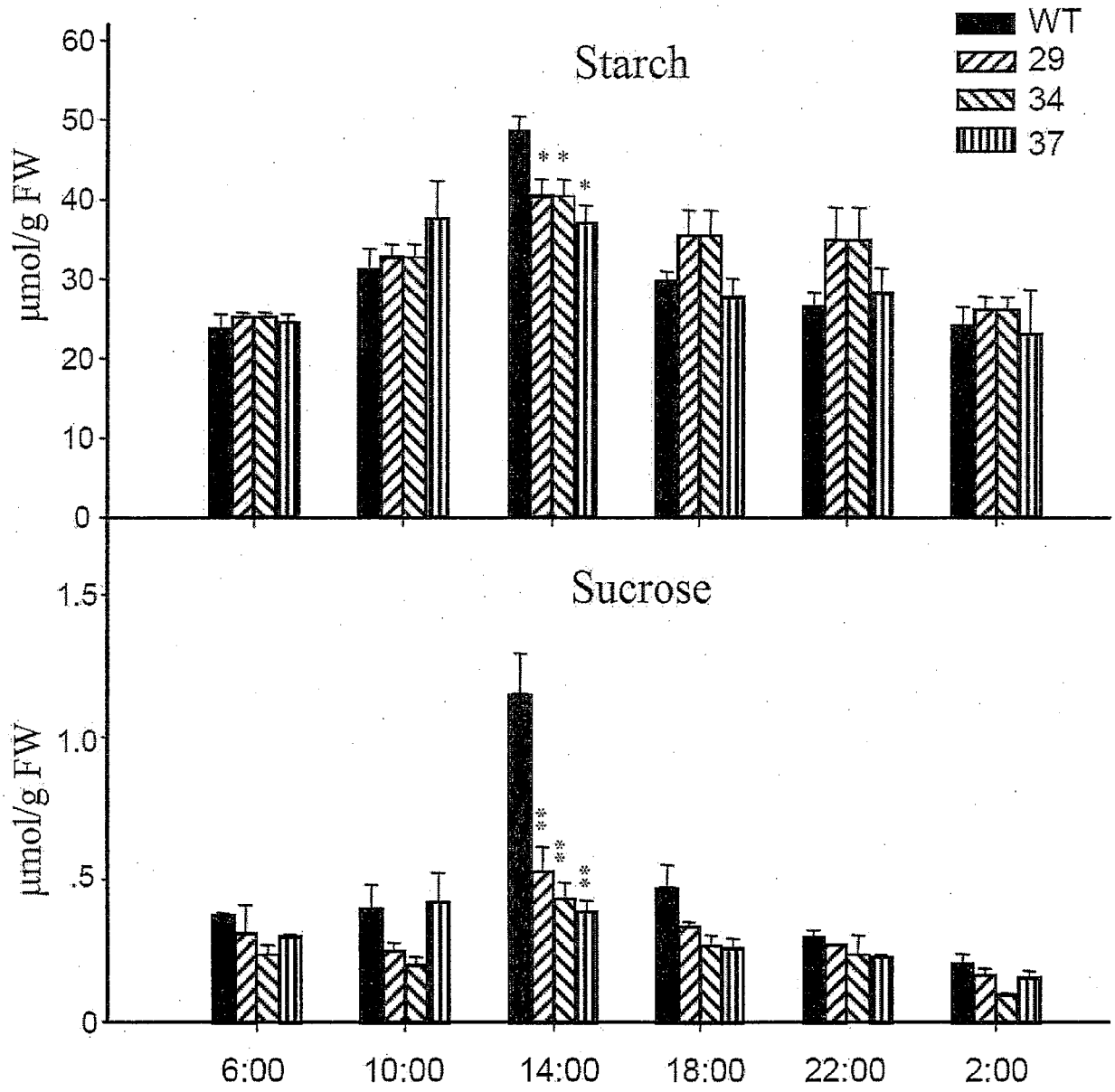

Starch
WT
29
34
37
µmol/g FW
60
50
40
30
20
10
0
Sucrose
µmol/g FW
1.5
1.0
.5
0
6:00
10:00
14:00
18:00
22:00
2:00

Figure 7

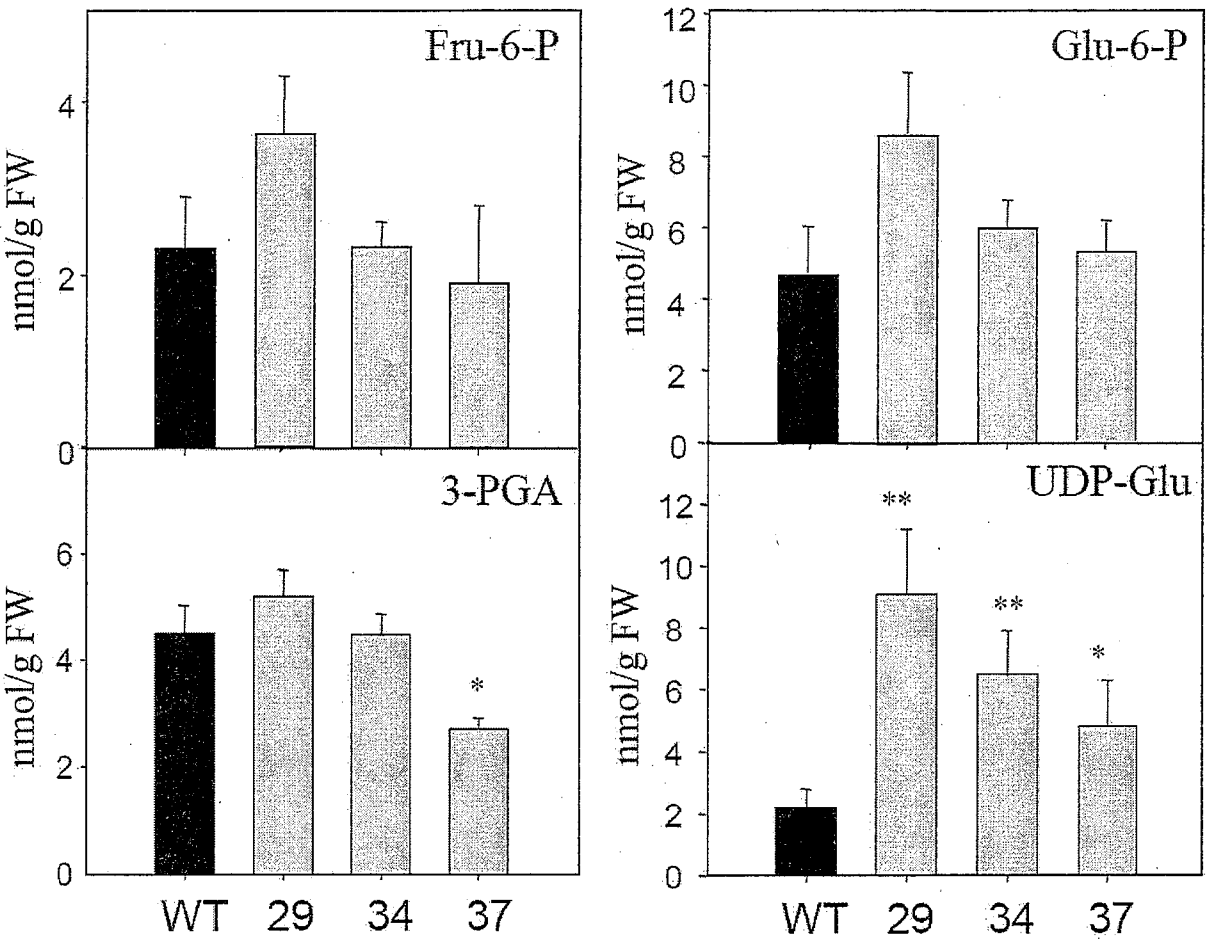
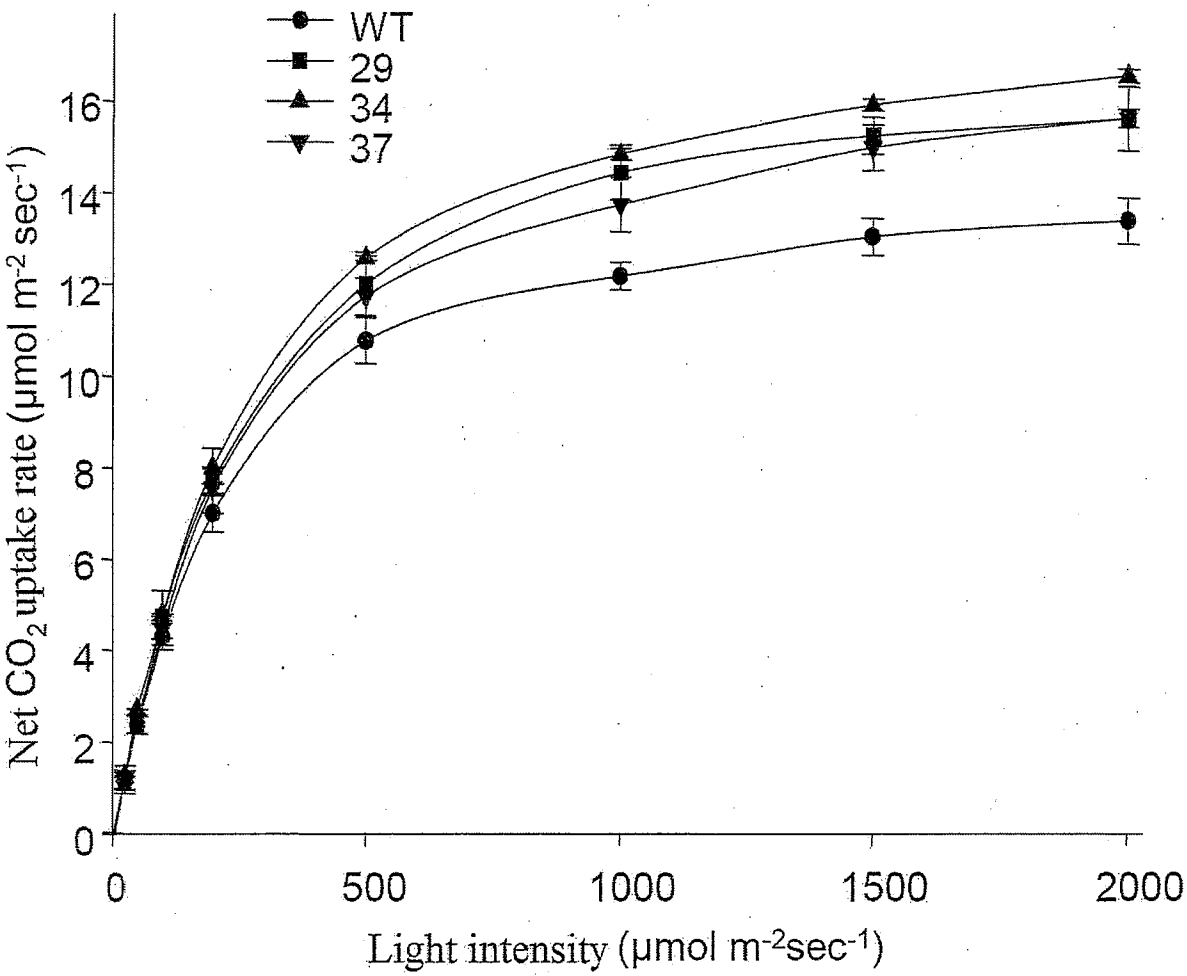

Fru-6-P
nmol/g FW
0
2
4
3-PGA
nmol/g FW
0
2
4
6
*
WT
29
34
37
Glu-6-P
nmol/g FW
0
2
4
6
8
10
12
UDP-Glu
nmol/g FW
0
2
4
6
8
10
12
**
**
*
WT
29
34
37

Figure 8


WT
29
34
37
Net CO_2 uptake rate (µmol m^{-2} sec^{-1})
0
2
4
6
8
10
12
14
16
0
500
1000
1500
2000
Light intensity (µmol $m^{-2}sec^{-1}$)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2008/055314

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC : C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases : EPODOC,WPI,PAJ,CNPAT,ISI WEB OF KNOWLEDGE ,CNKI

Search terms : pyrophosphatase, PPase, mesophyll, leaf, leaves, promoter, transgenic, transgene, transform

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US2007067873A1 (STEFFEN GREINER, et al.) 22 MAR. 2007(22.03.2007),see Example 4,Fig10A, claims 1,21	1-28
Y	Huang Dongjie, et al., Cloning of inorganic pyrophosphatase gene and construction of its plant expression vector. Chinese Journal of Tropical Crops, 30 June 2007, vol.28, no.6,pages 69-73,see page 69 first paragraph and discussion	1-28
Y	Si Lizhen, et al., Manipulation of Sucrose Synthesis in Transgenic Plants. Journal of Chinese Biotechnology, 31 January 2003,vol.23, no.1, pages 11-16,29,see page 15, left column last paragraph to page 16 left column first paragraph	1-28

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 APR.2009 (22.04.2009)	**07 May 2009 (07.05.2009)**
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451	Authorized officer XING ,Yunlong Telephone No. (86-10)62411094

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2008/055314

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CN1469705A (UNIV CONNECTICUT, et al.) 21 JAN. 2004(21.01.2004), see Claims 1-71	1-28
Y	J.-W. Lee,et al., Transgenic Arabidopsis plants expressing Escherichia coli pyrophosphatase display both altered carbon partitioning in their source leaves and reduced photosynthetic activity. Plant Cell Rep. 05 May 2005,vol.24,pages 374–382,see abstract, Fig 1 and discussion	1-28
A	CN1498966A (RES INST HEREDITY & DEVELOPMENTAL BIOLOGY) 26 May 2004(26.05.2004),see claims 1-10	1-28

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IB2008/055314

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
US2007067873A1	22.03.2007	WO2004083440A1	30.09.2004
		DE10313795A1	07.10.2004
CN1469705A	21.01.2004	WO0215674A1	28.02.2002
		AU5097401A	04.03.2002
		EP1315410A1	04.06.2003
		US2003213015A1	13.11.2003
		BRPI0113466A	17.02.2004
		NZ524616A	28.10.2005
		AU2001250974B2	21.06.2007
CN1498966A	26.05.2004	CN1238511C	25.01.2006

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2008/055314

Continuation of Box A:

IPC:

C12N15/63(2006.01) i
C12N15/82(2006.01) i
C12N15/29(2006.01) i
C12N5/04(2006.01) i
A01H5/00(2006.01) i